REMARKS

Claims 73-89 are pending in the application. The May 8, 2002 Office Action rejects claims 73-75 and 80-89 under 35 U.S.C. §112, first paragraph for failing to enable the entire scope of the claimed invention. Claims 73-89 have been under 35 U.S.C. §112, second paragraph as being indefinite. Applicant submits the following response to address the concerns stated in the Office Action. Applicants also submit concurrently herewith a Declaration of Dr. Mehmet Toner pursuant to 37 C.F.R. §1.132.

No new matter has been added. Amendment of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and has been done solely to more particularly point out and distinctly claim the invention, to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

The Final Restriction Requirement

Applicant is entitled to examination of at least groups III, IV, and V in this application.

> For the purposes of the initial requirement, a serious burden on the examiner may be *prima facie* shown if the examiner shows by appropriate explanation of separate classification, or separate status in the art, or a different field of search as defined in MPEP § 808.02. That *prima facie* showing may be rebutted by appropriate showings or evidence by the applicant. MPEP § 808.03 Restriction – When Proper.

To the extent that the Examiner has provided a *prima facie* case that the claims of Groups III, IV and V require independent searches because they require separate classification, Applicants rebut that *prima facie* case below.

According to the Examiner's restriction requirement, the inventions of each of the independent claims:

> are independent and distinct, each from the other. They have acquired a separate status in the art as a separate subject for inventive effect and require independent searches (as indicated by different classification). The search for each of the above inventions is not co-extensive particularly with regard to the literature search. Further, a reference which would anticipate one group would not necessarily anticipate or make obvious the other group.

This argument does not hold true. As even the Examiner has admitted, the inventions of groups III and V are of the same class and subclass: class 435, subclass 374. In fact class 435, subclass 374 ("Method of storing cells in a viable state") provides a fully adequate classification for each and every claim in the pending application.

The Examiner argues that the claims of Group IV (described as "drawn to a method for preserving nucleated cells having lipid membranes") should be classified in class 435, subclass 325 - a different classification than for Groups III and V. Class 435, subclass 374 (the classification for Groups III and V) fully describes the subject matter of the Group IV claims (the examiner describes the Group as "drawn to a method for preserving nucleated cells having lipid membranes" and 435/374 is directed to "Method of storing cells in a viable state"). The Examiner's proposed classification for the Group IV claims, class 435, subclass 325 (directed to "Animal cell, per se (e.g., cell lines, etc.); composition thereof; process of propagating, maintaining or preserving an animal cell or composition thereof; process of isolating or separating an animal cell or composition thereof; process of preparing a composition containing an animal cell; culture media therefore") is merely the parent classification to 435/374, and only includes irrelevant information with respect to the Group IV claims beyond that already included in 435/374 ("Method of storing cells in a viable state").

The Examiner implicitly recognized this in the parent case, which also included claims to preserving nucleated cells (see independent claims 1 and 10 of US Patent No. 6,127,177) just as the Group IV claims do; yet in the parent case, the Examiner classified the patent in 435/374; 435/1.3; 435/2; and the field of search was 435/374, 1.3, 2. The class/subclass

combination proposed by the Examiner for Group IV in the restriction requirement (435/325) was not searched and was not a classification for the parent patent despite the fact that its independent claims contain the same recitations as the Group IV claims. By the Examiner's own admissions and previous actions, Groups III, IV and V belong to the same class/subclass: 435/374. Accordingly, the Examiner's argument that independent searches are required does not hold.

In addition, in order to examine the Group IV claims, the Examiner will necessarily have to search the features that purportedly distinguish Group III and V claims from those of Group IV. For example, claim 76 (of Group IV which depends from independent claim 73) recites that the cell membranes are "porated using a membrane toxin" just as the Group V claims are. Similarly, claim 82 (of Group IV, depending from claim 73 through claims 81 and 80) requires that the bio-preservation agent consists essentially of a non-permeating sugar, just as the Group III claims do. Accordingly, searching Group III, IV and V claims together will not pose any additional burden on the Examiner.

Double Patenting Rejection

Applicant disagrees with the Examiner's Double Patenting rejection, however, in an effort to expedite prosecution of this application, Applicant includes with this Response a Terminal Disclaimer. The Terminal Disclaimer obviates the Double Patenting rejection made in the Office Action.

35 U.S.C. § 112 First Paragraph "Scope" Rejection

Claims 73-75 and 80-89 stand rejected under 35 U.S.C. § 112, first paragraph. In particular, the Examiner states that:

> [t]he specification, while being enabling for the method for preserving nucleated cells by "reversibly porating" the nucleated cells by a specific protocol encompassing the use of a "membrane toxin," *does not reasonably provide enablement for the method for preserving nucleated cells by a generic or unspecified protocol of reversible poration.* The *specification does not enable*

> any person skilled in the art to which it pertains, or with which it is most nearly connected, to the invention *commensurate in scope* with these claims. (emphasis added).

More specifically, the Examiner asserts that

> [a]mount of guidance and working examples are limited to the use of a "membrane toxin" . . . The use of a membrane toxin is the only example in the protocol intended for a step of "reversibly porating" membrane of the cellular material in the method for bio-preservation of cellular material.
>
> Applicants respectfully traverse this rejection.
>
> [T]he test of enablement is whether one reasonably skilled in the art could make or use the invention from the *disclosures in the patent coupled with information known in the art without undue experimentation.*" In fact, *a patent need not teach, and preferably omits, what is well known in the art. In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) (emphasis added).

The Examiner is correct to the extent that the working examples provided in the application use membrane toxins to reversibly porate cell membranes in order to load otherwise nonpermeable sugars pursuant to the invention. However, the guidance provided in the specification is broader than membrane toxins. So much so, that a person of ordinary skill in the art could readily rely on other prior art techniques that are well characterized for reversibly porating cell membranes for loading otherwise non-permeable materials into cells.

Guidance for Reversible Poration Provided in the Specification

For example, the specification provides at page 4, line 22 *et seq.* that the "target cells are then porated or permeabilized 20 to facilitate the loading of a bio-preservation solution. Preferably, the target cells are reversibly porated, that is pores are opened in the cell membranes of the target cells, but the poration process is controllably reversible . . ." Membrane toxins are then described as a porating agent "[i]n one

embodiment of the invention" (Page 4, line 27 *et seq.*), and the specification expressly states that "[o]ther poration agents may also be used with the method of the invention to reversibly porate target cells." (Page 5, line 19 *et seq.*).

In fact, Applicants have always characterized their invention as being able to work with any suitable reversible poration method. As originally filed, both in this application and in its parent (USSN 09/151,821, filed September 11, 1998), the first three independent claims provided methods that refer to the porating step simply as "reversibly porating the cell membranes." The discovery that drove Applicants' invention is that when cell membranes are reversibly porated and loaded with otherwise nonpermeable sugars for preservation, a number of unexpected and advantageous results can be achieved, such as the preservation of cells using only very low concentrations of sugar (resulting in simple loading procedures with no toxicity and no need to remove the cryoprotective agent), and the successful preservation of cells (namely nucleated cells) that otherwise have only been preserved using extremely complicated procedures, loading high levels of cyroprotective agents, and resulting in very little success. The particular method of reversible poration means nothing to Applicants' invention as long as it results in the desired level of intracellular sugar. Further supporting this conclusion is the fact that the membrane toxin method of reversible poration that is used in the examples was already well characterized in the prior art a the time of Applicants' filing (*see*, *e.g.*, the references cited at page 5, lines 1 through 14) and could not on its own contribute to the patentability of Applicants' invention.

Further guidance for performing the reversible poration step is provided in the application at page 6 where specific pore sizes are discussed, as well as the size of the preferred molecules, noting that 2nm diameter pores will allow molecules having a molecular weight of up to about 1000 to enter the target cells, thus allowing many

otherwise non-permeating bio-preservation agents to be used intracellularly. At pages 8 to 9, Applicants further discuss the use of reversible poration (any type of reversible poration) to improve loading of cryopreservation agents to decrease osmotic and toxic injuries to cells. Still further, Applicants describe at pages 13 to 14 how to determine whether reversible poration has succeeded – that is, how to tell whether the desired bio-preservation agent been correctly loaded intracellularly.

Reversible Poration is a "Textbook Procedure" Well Characterized in the Art

Techniques for porating cells, including reversible poration, are well characterized in the art – so much so that they are "textbook procedures." For example, the Biomedical Engineering Handbook[1] provides that:

> Bioelectric phenomena are of great interest and are ***well established*** topics in biomedical engineering. Electroporation is of growing interest because of its ability to rapidly and locally ***deliver molecules across bilayer membrane barriers***. Although initial applications have used in vitro cellular conditions and have focused almost exclusively on DNA introduction, the use of electroporation with tissue in vivo offers the prospect of "drug delivery" that can be electrically controlled. This is of great potential importance, because medical interventions are increasingly based on molecular rather than physical processes. ***Electroporation allows reversible or irreversible alteration of the cell membrane***, as well as other lipid-based barriers in tissues, such that the barriers to ions and molecules are reduced within microseconds by several orders of magnitude.

A person of ordinary skill in the art would recognize that this technique, well known before the filing date for the present application (this textbook was published in 1995), is perfectly suited to be used as the reversible poration step in the method of the present invention as it allows reversible poration specifically for delivering molecules across cell membranes – exactly as is required by the present invention.

[1] Bronzino, J. (Ed.), *The Biomedical Engineering Handbook*, Chapter 95 "Electroporation of Cells and Tissues," pages 1431 to 1440, CRC Press (1995) (hereinafter "Biomedical Engineering Handbook").

The Biomedical Engineering Handbook then goes on to characterize the amount of energy required to produce pores ranging in size from a radius of 1 nm (which is also the minimum size pore preferred for use with the invention) up to 20 nm (*Id.* at 1434) and how such pores can be made reversible or irreversible (*Id.* at 1437). Still further, the Handbook reports that, "[f]rom a biomedical engineering viewpoint, tremendously enhanced transport of molecules across the cell membrane (and tissues) is likely to be the most important feature of electroporation." (*Id.*) While the Handbook also reports that cell viability can suffer if pores are too large or if chemical exchange is too great, the instant application gives guidance on pore size and on low levels of cryoprotective agent to be loaded (in fact this is one of the great achievements of the present invention) so that a person of ordinary skill in the art could use electroporation or any other reversible poration technique with the invention to achieve success.

Reversible Poration Techniques Were Well Characterized in the Scientific Literature

Techniques for poration, reversible or irreversible, were routinely used in biological systems well before the application was filed. The introduction of holes or pores reversibly into the cell membrane involved either physical techniques such as electroporation, or biochemical techniques using salts, for example, calcium chloride, to alter the cell membrane. Typically, the formation of these pores in the cell membrane was to introduce exogenous DNA into a wide spectrum of host cells. However, the same techniques could also be used to introduce other exogenous compounds. There were a number of techniques that describe how to reversibly porate cell membranes to allow exogenous material to enter into the cells, as exemplified by a representative number of articles and abstracts provided herewith.

(A) Articles on Electroporation as a Technique for Reversible Poration

(i) Potter (1988) *Analytical Biochemistry* 174: 361-373. This review article provides examples of a number of different cell types that have successfully been

reversibly porated using an electric field, i.e., electroporation. These include mammalian cells, plant cells, unicellular organisms, as well as bacteria and fungi. The article also describes the introduction of proteins and small molecules by electroporation (*See* page 369, column 1).

(ii) Glogauer *et al.* (1992) *Exp Cell Res* 200:227-234. This article speaks to using electroporation as a method to gain access to the cell cytoplasm by transiently creating pores in cell membrane. Electroporation was used to introduce large-molecular-mass dextrans and proteins, as probes of the cytoplasmic compartment, into human fibroblast cells.

(iii) Saulis (1999) *Biomed Sci Instrum* 35:291-296, describes using electroporation to investigate the size and the apparent number of the pores induced in human red blood cells (i.e., nucleated cells) under the influence of a single electric field pulse. The data indicated that (i) the pores were small ($0.2 < \text{radius} < 0.5$ nm); (ii) the pores were of a size that permitted molecules of ascorbic acid and mannitol to penetrate through them when the field strength exceeded 2.5 kV/cm (tau i = 20 microseconds); (iii) with increasing pulse intensity there was an increase in both the radius of the pores and the number of cells permeable to mannitol or sucrose. It was also concluded that the presence of only one small (radius approximately 0.3 nm) pore in the human erythrocyte was sufficient for a cell to be regarded as porated.

(iv) Chang *et al.* (1990) *Biophys J* 58:1-12. This article shows the effect of electroporation on the cell membrane by using rapid-freeze electron microscopy, in particular, to examine human red blood cells. Volcano-shaped membrane openings appeared in the freeze-fracture faces of electropermeabilized cell membranes at intervals as short as 3 milliseconds after the electrical pulse. These openings represent the membrane pathways which allow entry of macromolecules (such as DNA) during

electroporation. The pore structures rapidly expand to 20-120 nm in diameter during the first 20 milliseconds of electroporation, and after several seconds begin to shrink and reseal.

(B) Glass beads for reversible poration

In addition, at the time the invention was filed, electroporation was by no means the only method available for reversible poration. In fact, reversible poration could be performed by using glass beads, as described below:

(v) Fennell *et al.* (1991) *Arterioscler Thromb* 11:97-106, describes an alternative method of porating the cell membrane by incubating endothelial cells (ECs) with glass beads, which resulted in permeabilization of human and bovine ECs. This poration of the plasma membrane allowed the introduction of macromolecules sucah as dextrans less than or equal to 152 kilodaltons, and immunoglobulins, as well as small, charged molecules (e.g., Lucifer Yellow). The nonspecific permeabilization of the EC was transient and the integrity of the plasma membrane was reestablished. The permeabilized ECs also regained functional characteristics.

(C) Pore-forming toxins for reversible poration

Another alternative method for reversible poration relied on using pore-forming toxins. Applicants submits that at the time the application was filed, poration of cellular membranes with specialized proteins was well known in the art. Examples of pore-forming proteins include C5b-9 complex of the compliment, the cystolysin of cytotoxic T lymphocytes and various exotoxins prodced by several strains of *Staphylococcus* and *Streptococcus*. Thus, the art was replete with teachings for using a number of membrane toxins, as exemplified below:

(vi) Ahnert-Hilger *et al.* (1989) *Methods Cell Biol* 31:63-90. This article provides a general review of cell poration, as well as an analysis of pore-forming toxins such as α-toxin from *Staphylococcus aureus*, and streptolysin O (SLO) from α-hemolytic sterptococci. The teachings of Ahnert-Hilger *et al.* show that α-toxins permeabilize cells for low molecular weigh substances, while SLO permeabilize cells for both high and low molecular weight substances.

(vii) Thelestam *et al.* (1988) *Toxicon* 26:55-65. This article provides a general review describing the advances in Staphylococcal alpha toxin. In particular, about the basic biochemistry and interaction of this toxin with membranes, and the concept of alpha toxin as a channel forming protein.

(viii) Harvey *et al.* (1994) *Mutat Res* 315:17-23. This reference uses CHO (Chinese hamster ovary), xrs5 (X-ray sensitive Chinese hamster) and HF19 (untransformed human fibroblast) cells, which were exposed to a lethal dose of the restriction enzyme Pvu II during electroporation or poration with the bacterial toxin streptolysin O. The results showed that electroporation alone proved to be more cytotoxic to the cells, whilst streptolysin O was more efficient at permeabilizing both hamster and human cells.

(ix) Bhakdi *et al.* (1978) *Proc Natl Acad Sci U S A* 75:5655-9. This reference reports on the ability of the pore-forming protein, purified C5b-9 complex, isolated from target membranes to become reincorporated into artificial lipid vesicles. The data indicates that the complex has a vertically oriented, hollow, cylindrical macromolecule possessing lipid-binding regions, that enables one terminus to penetrate into the lipid bilayer. A transmembrane pore appears to be created at the attachment site of the C5b-9 complex.

(x) Masson *et al.* (1985) *J Biol Chem* 260:9069-72 describe the isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. The isolated perforin polymerized and inserted into lipid bilayers in the presence of Ca^{2+}, forming tubular structures with inner diameters varying from 6 to 16 nm.

Collectively, these articles and abstracts demonstrate that the use of reversible poration to introduce pores into a cell membrane was well established, well known, and well characterized, at the time the invention was filed. There were a number of different ways in which the reversible poration could be conducted. Thus, a skilled artisan in the field would readily be able to reversibly porate a lipid membrane of a cell using mere routine experimentation, rather than undue experimentation, based not only on the teaching in the specification, but also on the ample knowledge available in the art for performing such techniques.

Applicants also submit concurrently herewith a Declaration of Dr. Mehmet Toner pursuant to 37 C.F.R. §1.132 to further overcome these rejections. This Declaration establishes that one skilled in the art would be able to use the application's disclosure, in addition to the knowledge available in the art, to apply the invention to reversibly porating cells with any number of reversible porating techniques available at the time the invention was filed.

The above articles also address the Examiner's assertion that the:

> [s]pecification does not provide a teaching or suggestion drawn to other than either generic 'membrane toxin' or particular *Staphylococcus aureus* alpha toxin. The use of a membrane toxin is the only example in the protocol intended for a step of 'reversibly porating' membrane of the cellular material in the method for bio-preservation of cellular material.

Applicants submit that using pore-forming proteins was well known in the art, as evidenced by the representative number of references cited above (articles vi-viii).

Thus, one skilled in the art could have practiced the invention, using not only the α-toxin disclosed in the specification, but any number of other toxins that were used, and available in the art at the time the specification was filed.

Applicants' Own Laboratory Work Shows That Reversible Poration Techniques Known in the Art Can be Used With the Invention Without 'Undue' Experimentation

Applicants provide further evidence that porating cells was well characterized, and could be routinely performed using any number of porating techniques available at the time the application was filed without undue experimentation, to address the Examiner's concern that:

> [t]he neither specification nor the prior art can be said to support the enablement of the claims over their breath. Thus, it is considered that *undue experimentation* would be required to practice the invention as claimed due to the amount of experimentation necessary because of the limited amount of guidance and limited number of working examples in the specification with regard to a generic concept of reversible poration.

Applicants submit that a determination of whether the extent of experimentation is undue, is based upon a *balanced analysis* of many factors, referred to as the Wands factors which include, but are not limited to the following:

(A) The breadth of the claims;

(B) The nature of the invention;

(C) The state of the prior art;

(D) The level of one of ordinary skill;

(E) The level of predictability in the art;

(F) The amount of direction provided by the inventor;

(G) The existence of working examples; and

(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

When making a determination that a specification lacks enablement and would require undue experimentation to practice the claimed invention, all of these factors have to be analyzed together, it is improper to analyze only one of the factors, as stated below:

> [I]t is *improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others*. The examiner's analysis must consider all the evidence related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. 858 F.2d at 737, 740, 8 USPQ2d at 1404, 1407. (emphasis added).

It is Applicants' position that the Examiner has only considered one of the Wands factors *i.e.*, the breadth of the claims, without considering the others. In fact, when all of the other factors are considered and balanced, the amount of experimentation would not be considered to be undue experimentation. For example, the breadth of claims is certainly commensurate to the scope of disclosure for all the reasons outlined above. Also, the state of the prior art at the time the application was filed, was replete with teachings of how to reversibly porate cells by any number of different methods, as evidenced by the articles provided. Furthermore, the level of skill of an ordinary artisan was fairly sophisticated, any ordinary artisan in the field of molecular biology could reversibly porate cells using routine experimentation, and in fact, were reversibly porating cells to introduce exogenous materials well before the filing date of the invention. Furthermore, the art was quite predictable, it was well established that applying electric fields to various cell types, or the use of pore-forming toxins would generate pores in a cell membrane. In addition, the specification provides ample guidance, as well as working examples of the invention. Thus, based on a the specification and the knowledge available in the art, as well as a closer, balanced examination of the Wands factors, Applicants submit that the claimed invention is enabled and can be practiced without undue experimentation.

As further support that reversible poration is a standard routine technique, that can be practiced without undue experimentation, and that a person of ordinary skill in the art would have a number of other resources available to him or her, to select and use a reversible poration technique for use with the claimed invention, Applicants have relied on Professor James Weaver's chapter on "Electroporation of Cells and Tissues" available in The Biomedical Engineering Handbook (CRC Press, Bronzino, J. (Ed.) (1995)) to show that electroporation was one form of reversible poration that could readily be used in the claimed invention by the skilled artisan, without undue experimentation. Using the method outlined by Professor James Weaver also demonstrates that any method of reversibly porating a cell available to the skilled artisan can be employed, not just the method disclosed by Applicants in the specification.

Relying on Professor James Weaver's description of electroporation, Applicants have successfully demonstrated the reversible poration of cells without undue experimentation (*See* Declaration of Inventor, filed herewith). Paragraph 8 of the Declaration clearly states that a reversible poration technique within the claimed invention could be performed without undue experimentation. The data generated shows that human foreskin fibroblast cells are porated using electroporation, as evidenced by the uptake of the fluorescent marker, propidium iodide (PI). This work shows that reversible poration can readily be accomplished within the scope of the invention without undue experimentation.

Furthermore, Paragraph 11 of the Declaration, and Fig. 3 clearly demonstrate that the reversibly porated cells could be loaded with the preferred sugar (trehalose), at a preferred concentration (0.2M) required to practice the invention, without undue experimentation.

For all the foregoing reasons, it is Applicants' position that the specification as filed had sufficiently enabled the claimed invention. Accordingly, the Examiner is respectfully requested to withdraw the rejection.

35 U.S.C. § 112 Second Paragraph Rejection

Claims 73-89 have been rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In particular, the Examiner states that:

> [c]laim 73 is indefinite because it appears to lack an antecedent basis for mammalian cells in step d) as claimed. . . it appears to lack antecedent basis for 'the cellular material' in step b) wherein it is not particularly clear whether the material is loaded is the same material which is known as a control for determination of a 'sufficient' concentration. ... the phrase 'cryopreserving' in step c) of claim 73 contains a typographical error.

In response, Applicants have amended claim 73 to recite a "the nucleated cells" in step (d), for which antecedent basis is present in the preamble of the claim. The claim has also been amended to recite "a cellular material" in step (b) and reference to the term "sufficient" has been removed form the claim. In addition, the typographical error has been corrected. Accordingly, the rejections with regard to this claim are rendered moot.

Claim 74 has been rejected as being indefinite because it lacks proper antecedent basis for the phrase "cellular material."

In response, Applicants have amended claim 74 to recite "nucleated cells" for which antecedent basis is provided in independent claim 73, thereby rendering the rejection moot.

Claims 83-85 have been rejected as being indefinite because they appear to lack a proper antecedent basis for "mammalian cells."

In response, Applicants have amended claim 83-87 to recite "nucleated cells" for which antecedent basis is provided in independent claim 73. Claim 85 has also been amended to remove reference to the term "sufficient" to be consistent with the removal of this term from claim 73. Accordingly, the rejection is rendered moot with regard to these claims.

Applicants have also amended claim 82 and 88 to correct a typographical error to change the dependency of these claims such that they depend from claim 80 rather than claim 81.

Conclusion

For all of the foregoing reasons, Applicants submit that the claims are enabled and are in condition for allowance. Applicants respectfully request a notice of allowance for these claims. Applicants further request that the Examiner telephone the undersigned Attorney for Applicants in the event that such communication might expedite prosecution of this matter.

Respectfully submitted,

Date: June 13, 2003

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